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Effect of Salt Stress in the Selection of Salt Tolerant Hybrids in Rice (*Oryza sativa* L.) Under *in vitro* and *in vivo* Condition

S. Babu, A. Sheeba, P. Yogameenakshi, J. Anbumalarmathi and P. Rangasamy
Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai 625 104, Tamil Nadu, India

Abstract: In the present study, the salt tolerant genotypes were selected in both *in vitro* and *in vivo* condition. For *in vivo* condition all the genotypes were raised in three different salt affected areas having different soil and water pH and EC and observations were made on leaf proline content, Na^+:K^+ ratio, chlorophyll stability index and single plant yield. The following hybrids TS 6/TRY 1, TS 6/BTS 24, TS 29/CSR 27, TS 29/BTS 24, IR 58025 A/TRY 1, IR 58025 A/BTS 24 were stable for most of the traits in all the environments. Under *in vitro* condition, the response of plant cell to salt stress and the feasibility of selecting salt tolerant callus were studied. Callus was grown on agar solidified media containing 0.2, 0.4, 0.6, 0.8 and 1.0% NaCl salt. Parameters such as fresh weight of callus, callus morphology and proline content were studied. The callus growth decreased with increasing NaCl concentration in the medium. TS6/BTS 24, TS 6/Vytilla 1, TS 6/TRY 1, TS 29/BTS 24 and IR 58025 A/Vytilla 1 were the hybrids with high tolerance to salt stress *in vitro*.

Key words: *Oryza sativa*, rice, callus, salt tolerance, environments

INTRODUCTION

The threats of hunger loom large in Asia due to the declining rice production growth rate in recent years. Salinity is a serious problem affecting one third of the irrigated land (Mass and Hoffman, 1977) and limiting the yield potential of modern cultivars. It has been estimated that salts affected nearly 950 million ha of land in the world. In India it is estimated that 8.6 million ha (Pathak, 2000) of land area is highly prone to salts. By manipulating the heritable variation present in the germplasm, we can develop saline tolerant cultivars through breeding techniques, but it is a cumbersome and time-consuming process. The slow progress is due to the complexity of the problem involving salinity in the soil and the genetic system. Development of salt tolerant varieties has gained momentum among the breeders in the recent past. Development of hybrid rice with inbuilt salinity tolerance in most desirable to increase the production capacity of rice under saline condition. Tissue culture technique offers yet another tool in developing stress tolerant somaclonal variants (Nabors and Dykes, 1985). In view of the economic importance and also the low salt tolerance shown by rice when compared to other cereals, the present study was undertaken to understand the *in vitro* and *in vivo* behavior of rice cultivars in salt affected environments.

MATERIALS AND METHODS

Four male sterile lines viz., TS 6 (L_1), TS 29 (L_2), COMS 9A (L_3) and IR 58025A (L_4) were used as lines and nine varieties known for salt tolerance namely CSR 13 (T_1), CSR 27 (T_2), Pokkali (T_3), Vytilla 1 (T_4), TRY 1 (T_5), CO 43 (T_6), Jaya (T_7), BTS 24 (T_8) and Vytilla 2 (T_9) were used as testers and their hybrids were taken for the study. For *in vivo* condition all the parents and resultant hybrids were raised in three different salt affected areas like Anbil Dharmalingam Agricultural College and Research Institute, Trichy (E_1) with soil and irrigation water EC of 7.25 and 1.84 ds m^{-1}, respectively and soil and water pH of 9.2 and 7.6, respectively; Pandit Jawaharlal Nehru College of Agriculture and Research Institute, Karaikal (E_2) with soil and irrigation water EC of 4.81 and 0.86 ds m^{-1}, respectively and soil and water pH of 8.4 and 7.2, respectively and farmer's field, Konthagai-Elanthakulum, Madurai (E_3)with soil and irrigation water EC of 4.26 and 2.38 ds m^{-1}, respectively and soil and water pH of 8.2 and 7.8, respectively during *Kharif*-2002. The experiment was laid out in a randomized block design with two replications and plants were raised at a spacing of 20×15 cm. Observations were made on five randomly selected plants per replication for salt stress related traits namely leaf proline content, Na^+:K^+ ratio and chlorophyll stability index along with single plant yield.

Corresponding Author: S. Babu, Botany Division, UPASI Experimental Farm, UPASI Tea Research Foundation, Tea Research institute, Nirar Dam B. P. O., Valparai-642 127, Coimbatore Dist. Tamil Nadu, India
Tel: 04253-235 301, 235 303 Fax: 04253-235302

The *in vitro* response of genotypes was studied in Tissue Culture Laboratory in the Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai during 2002. The explant used in the research was dehulled mature hybrid and parental seeds (mature embryo). The seeds were sterilized in 70% ethanol for one minute, followed by 0.1% mercuric chloride for eight minutes and washed three times with sterile distilled water to remove the excess chemicals. The seeds were placed in MS (Murashige and Skoog, 1962) callus induction medium with three different combination of growth regulators viz., MS + 2,4-D 2 mg L^{-1}; MS + 2, 4-D 2 mg L^{-1} + Casein hydrolysate 1 g L^{-1}; MS + 2, 4-D 3 mg L^{-1} + Casein hydrolysates 1 g L^{-1}. After 21 days of culturing, the callus formed was subcultured to fresh callus proliferation medium without 2, 4-D to effect more callus growth. Small pieces of uniformed size calli were transferred to salt stress medium containing MS medium+ 2, 4-D mg L^{-1} + kinetin 0.25 mg L^{-1} along with five different concentrations of NaCl salt (0.2, 0.4, 0.6, 0.8 and 1.0%) in order to screen the salt tolerant calli. The calli were left for a month to proliferate at 26±2°C. After proliferation the increased calli growth was estimated as fresh weight of callus on salinized medium and expressed in milligram.

After the end of callus proliferation period in all the concentrations, the callus morphology was scored and recorded. For scoring a rating scale of 1-9 was used (Pushpam and Rangasamy, 2000) as mentioned below

Score	Description of callus
1	Completely turned block or dark brown, dead
3	Watery/sticky appearance, more than 75% of callus turned brown
5	Yellow to brown in colour, water soaked with slimy surface
7	Yellow to pale yellow, water soaked areas interspersed with pale yellow friable callus
9	Pale yellow to white in colour, healthy, nodular and friable

At the end of callus morphology scoring the calli at 0.2 (low) and 1.0% (high) NaCl concentrations were taken and subjected to proline accumulation test. The estimation was carried out by the method suggested by Bates *et al*. (1973) and expressed in µg g^{-1} of callus. The analysis was done appropriate for the factorial RBD.

RESULTS

For field studies: The mean values for the characters leaf proline content, Na^+:K^+ ratio, chlorophyll stability index and single plant yield under individual and pooled environments were recorded. Based on the performance of parents and hybrids under different saline environments, the parents and hybrids showing high *per se* for these traits studied were grouped and given in the Table 1.

For *in vitro* studies: Salt stress affected the growth of callus development. The callus growth decreased with increase in salt concentrations.

The interaction effect of lines, testers and hybrids with NaCl concentration for fresh weight of callus showed that TS 29 (94.70 mg) at 0.2% level was good followed by IR 58025 A (84.81 mg) in the same level whereas, IR 58025 A (34.33 mg) and TS 29 (31.46 mg) showed high callus weight at 1% level. The testers, BTS 24 (110.69 mg), TRY 1 (110.04 mg), Vytilla 1 (105.77 mg) and Pokkali (101.19 mg) at 0.4% level produced more fresh callus weight. Vytilla 2 (25.58 mg) and CSR 27 (25.13 mg) at 1% NaCl produced less fresh weight of callus and found that the interaction was very high but the interaction was low at 1% level in testers TRY 1 (47.85 mg) and BTS 24 (45.75 mg). The interaction effect of hybrids showed that TS 29/BTS 24 (125.31 mg), IR 58025 A/Vytilla 1 (114.30 mg) and IR 58025 A/Pokkali (111.74 mg) found least interaction with NaCl concentration and the hybrids COMS 9A/Jaya (16.29 mg), COMS 9A/CO 43 (18.99 mg) and TS 29/Jaya (20.27 mg) were observed to have high interaction effect at 1% NaCl concentration.

Table 1: Genotypes showing high *per se* for different characters under different environment and pooled condition

Characters	E_1	E_2	E_3	Pooled
Leaf proline content (mg g^{-1})	COMS 9A (0.78), BTS 24 (0.99), TS 29 / BTS 24 (1.08)	COMS 9A (0.88), BTS 24 (1.04), TS 29/BTS 24 (1.30)	TS 29 (0.67), BTS 24 (0.81), TS 29/BTS 24 (1.08)	TS 29 (0.77), BTS 24 (0.94), TS 29/BTS 24 (1.15)
Na^+:K^+ ratio	TS 6(0.41), Vytilla 2(0.34), TS 29/BTS 24 (0.31)	TS 29(0.36), TRY 1 (0.29), TS 6/Vytilla1 (0.20)	TS 29 and IR 58025 A (0.36), Pokkali (0.25), TS 29/BTS 24, COMS 9A /Vytilla 1 and TS 6/TRY 1 0.23	TS 29 and IR 58025A (0.39), BTS 24(0.32), TS 29/ BTS 24, TS 6/BTS 24 and TS 6/Vytilla 1 (0.28)
Chlorophyll stability index	Vytilla 1(42.0), Pokkali (56.0), TS 29/ Vytilla 2 (88.4)	IR 58025 A(46.9), BTS 24 (68.5), IR58025A/BTS 24 (78.7)	TS 29 (72.1), Pokalli (87.8), TS 29/BTS 24 (92.4)	IR 58025 A (50.4), Pokalli (70.5), IR 58025A/ BTS 24 (82.8)
Single plant yield (gram)	TS 29 (14.2), BTS 24 (13.9), TS 29/BTS 24 (19.0)	TS 29 (10.9), TRY 1 (13.1), TS 6/CO 43 (19.4)	TS 29 (11.1), BTS 24 (15.0), TS 29/BTS 24 (22.4)	TS 29 (12.1), BTS 24 (13.8), TS 29/BTS 24 (20.3)

The estimates of interaction between lines and NaCl concentrations for the trait callus morphology expressed that TS 29 (1.82) and TS 6 (1.37) registered high callus morphology score at 1.0% NaCl. Among testers, BTS 24 (7.21, 3.39), TRY 1 (7.04, 3.16) and Vytilla 1 (6.9, 3.13) had least interaction with 0.2% NaCl and 1% NaCl concentrations, respectively and said to be salt tolerant genotypes. The high callus morphology score was recorded by the hybrids TS 29/BTS 24 (7.59, 2.66) at 0.2% and 1% NaCl followed by TS 6/BTS 24 (2.49) at 1% level. For the trait proline content, the interaction inferred that at 1% NaCl, TS 29 (330.00 $\mu g\ g^{-1}$) and TS 6 (313.50 $\mu g\ g^{-1}$) recorded high proline accumulation. Similarly, BTS 24 (367.75 $\mu g\ g^{-1}$) followed by TRY 1 (304.75 $\mu g\ g^{-1}$) synthesized high proline content at 1% NaCl showed these genotypes were said to be tolerant and least effective to salt concentrations. In the hybrids, COMS 9A/CO 43 (158.00 $\mu g\ g^{-1}$) recorded less proline content at 0.2% NaCl while TS 29/BTS 24 (611.00 $\mu g\ g^{-1}$), TS 6/BTS 24 (462.00 $\mu g\ g^{-1}$) and TS 6/TRY 1 (458.50 $\mu g\ g^{-1}$) registered high proline content at 1% NaCl level.

DISCUSSION

Rice varieties differ in their response to salt stress and yielding ability. Comparison of yield of genotypes under various stress situations is obviously essential to define their genetic potentiality as well as to study the reasons for their better yield owing to tolerance. In the present case, the parents and hybrids were screened based on their performance (in terms of high *per se* and heterosis) under different saline environment for the characters studied. Selection of genotypes with high *per se* was the important objective among the breeders for the improvement of yield and other traits in a breeding programme.

Based on the *per se* performance of parents for different environments TS 29 registered significant *per se* values for leaf proline content, chlorophyll stability index and single plant yield. Among testers BTS 24 recorded highly significant mean values for all the characters studied followed by TRY 1 for leaf proline content, Na^+:K^+ ratio and single plant yield. Hence, TS 29, BTS 24 and TRY 1 were identified as better parents since they exhibited good mean performance for most of the traits over environments. In the case of hybrids, TS 29/BTS 24, TS 6/Jaya, TS 6/BTS 24, IR 58025 A/Vytilla 1, IR 58025 A/BTS 24 and TS 6/Pokkali were identified as best hybrids as they recorded high *per se* values for characters related to salt stress like leaf proline content, Na^+:K^+ ratio and chlorophyll stability index besides high single plant yield.

The percentage of heterosis expressed by the hybrids for different characters over the standard parent CORH 2 is presented in Table 2.

Table 2: Standard Heterosis percentage over CORH 2 for different characters (under pooled condition)

Hybrids	Leaf proline content	Chlorophyll Na^+:K^+ ratio	Single Plant Stability Index	Yield
L_1 x T_1	20.7*	-17.5*	18.7*	7.0*
L_1 x T_2	67.2*	-15.0*	29.1*	12.2*
L_1 x T_3	72.4*	-15.0*	33.3*	15.5*
L_1 x T_4	51.7*	-30.0*	25.9*	0.5
L_1 x T_5	22.4*	-27.5*	29.8*	-2.1
L_1 x T_6	36.2*	-17.5*	9.5	-1.2
L_1 x T_7	29.3*	-15.0*	8.3	-11.2*
L_1 x T_8	60.3*	-30.0*	39.2*	13.2*
L_1 x T_9	43.1*	-12.5*	12.9*	-13.6*
L_2 x T_1	25.9*	-22.5*	31.7*	-13.7*
L_2 x T_2	12.1*	-25.0*	48.0*	-6.6*
L_2 x T_3	25.9*	-25.0*	52.5*	-7.6*
L_2 x T_4	24.1*	-22.5*	56.5*	-1.7
L_2 x T_5	20.7*	-27.5*	45.4*	-7.6*
L_2 x T_6	24.1*	-15.0*	33.4*	-11.2*
L_2 x T_7	44.8*	-15.0*	47.3*	10.9*
L_2 x T_8	98.3*	-30.0*	69.9*	26.9*
L_2 x T_9	32.8*	-10.0*	46.2*	-13.9*
L_3 x T_1	-8.6	-17.5*	-5.9	-45.4*
L_3 x T_2	-3.4	-7.5	2.8	-58.1*
L_3 x T_3	-3.4	-12.5*	14.1*	-55.4*
L_3 x T_4	6.9	-22.5*	-4.1	-61.3*
L_3 x T_5	6.9	-5.0	2.6	-61.0*
L_3 x T_6	8.6	-10.0*	-5.9	-60.2*
L_3 x T_7	-1.7	-5.0	11.7*	-59.6*
L_3 x T_8	12.1*	-20.0*	40.4*	-57.8*
L_3 x T_9	17.2*	-15.0*	-1.6	-58.8*
L_4 x T_1	51.7*	-12.5*	28.9*	1.9
L_4 x T_2	20.7*	-17.5*	33.7*	-10.0*
L_4 x T_3	17.2*	-25.0*	40.0*	3.1
L_4 x T_4	50.0*	-25.0*	53.1*	7.7*
L_4 x T_5	31.0*	-17.5*	54.2*	2.1
L_4 x T_6	34.5*	-7.5	39.6*	-15.9*
L_4 x T_7	41.4*	-12.5*	41.8*	-0.4
L_4 x T_8	48.3*	-27.5*	33.8*	4.4
L_4 x T_9	56.9*	-17.5*	49.1*	-4.7
SE	0.03	0.02	1.49	0.53

* Significant at 5% level

The hybrids TS 6/CSR 13, TS 6/CSR 27, TS 6/BTS 24, TS 29/Jaya, TS 29/BTS 24 and IR 58025 A/Vytilla 1 registered significantly positive standard heterosis values for single plant yield. These hybrids also registered highly significant positive heterosis value for leaf proline content and chlorophyll stability index and negatively high heterosis for Na^+:K^+ ratio and hence could be adjudged as best hybrids for the improvement of salt tolerance along with high yield. Combining both mean performance and heterosis percent TS 6/BTS 24, TS 29/BTS 24 and IR 58025 A/Vytilla 1 are highly suitable for saline prone conditions and could be recommended for heterosis breeding coupled with salt tolerance capacity.

Though the traditional methods of breeding have resulted in many tolerant varieties, the potential of the novel technique to develop tolerant varieties through *in vitro* selection cannot be left unnoticed. So the study was undertaken to combine the *in vitro* tissue culture response of genotypes along with their *in vivo* response.

In the present investigation, different rice cultivars responded differently to various levels of NaCl concentration for fresh callus weight. Resistant varieties showed minimum reduction in callus weight even at high level of NaCl i.e., at one per cent. This suggests that genotypic constitution plays a major role in callus development (Pushpam and Sree Rangasamy, 2000). The hybrids evolved from the tolerant cultivars also produced fresher callus at high level of NaCl. Generally there was decrease in callus weight with increase in concentration of NaCl irrespective of parents and hybrids. The parents IR 58025 A, TS 29, TRY 1, BTS 24 and Vytilla 1 produced considerable amount of callus at one percent NaCl. Among the hybrids TS 29/BTS 24 followed by IR 58025 A/Vytilla 1, IR 58025 A/Pokklai, TS 6/Vytilla 1, TS 6/CSR 27, TS 6/CSR 13 and TS 6/TRY 1 recorded high callus weight at 0.2 and 1.0% NaCl (Fig. 1). The exposure of callus to a saline environment may lead to water stress and specific ionic imbalance perhaps resulting in ion toxicity. Further more, cell grown under stress may have to spend more metabolic energy than those grown in the absence of stress. The extra energy most probably is used up in regulating osmotic adjustment resulting in decline in fresh weight.

The morphology of callus is taken as a criterion for selection of callus for regeneration under stress condition (Nabors and Dykes, 1985; Narayanan and Sree Rangasamy, 1991). For scoring a rating scale of 1-9 was used. The scoring was made in all the levels of NaCl. Generally there was a decrease in callus growth and score with increase in the salt concentration. Among the parents, IR 58025 A and COMS 9A were least responsive while L_2 registered high score. Among Testers BTS 24, TRY 1, Vytilla 1 and Pokkali registered high callus morphology scores at 0.2 as well as 1.0% NaCl levels. A series of high scores were recorded by the hybrids TS 29/BTS 24, TS 6/BTS 24, TS 29/Pokkali, TS 29/Vytilla 2, COMS 9A/Vytilla 1, TS 29/Vytilla 1 and COMS 9A/BTS 24 and very low score was noticed in TS 6/CSR 13, IR 58025 A/CSR 13 and TS 29/CSR 13 at one percent NaCl concentration (Fig. 2). This inferred that these hybrids are tolerant and susceptible to high salt concentrations, respectively (Pusphpam and Sree Rangasamy, 2000).

Proline content increased linearly with increasing salinity in both parents and hybrids. Higher proline level in salt adopted callus may be due to an increased rate of synthesis or decreased rate of oxidation of this compound (Wyn Jones and Gorham, 1984). The accumulation of organic solutes like proline has been implicated in salt tolerance mechanisms in many plant species including rice (Chandler and Thorpe, 1987). In the present study the proline content increased invariably to increased NaCl concentration. Among the parents TS 29, TS 6, BTS 24, TRY 1, Vytilla 1 and Pokkali registered higher proline content at 0.2 and 1.0% level of NaCl. Those hybrids accumulated high proline at 1.0% level were TS 29/BTS 24, TS 6/BTS 24, TS 6/Vytilla 1, TS 6/TRY 1, TS 29/Pokkali, TS 29/TRY 1, IR 58025 A/Vytilla 1 and IR 58025 A/TRY respectively (Fig. 3). There were divergent reports related to role of proline in salt tolerance was observed by

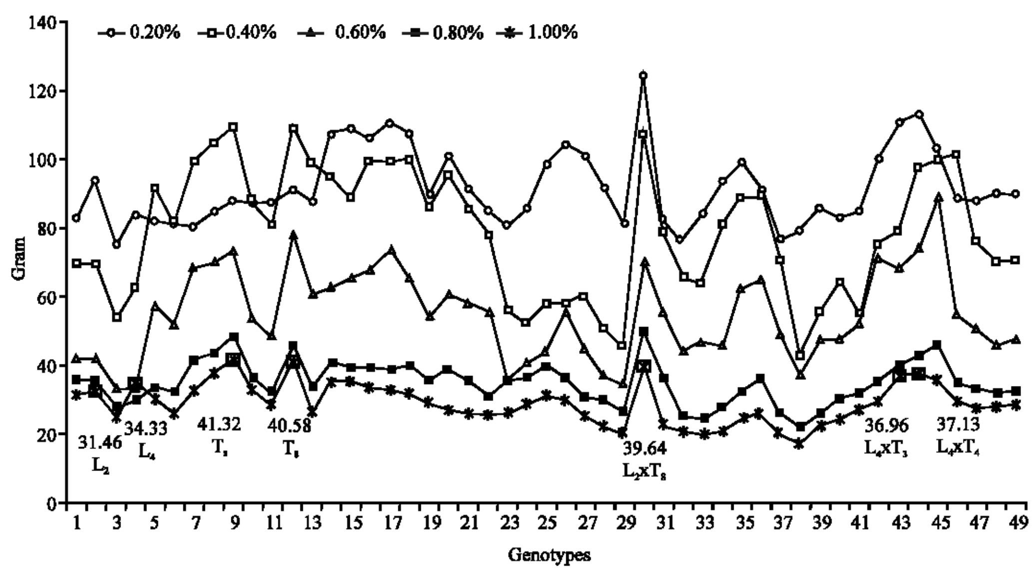

Fig. 1: Effect of NaCl on fresh weight of callus

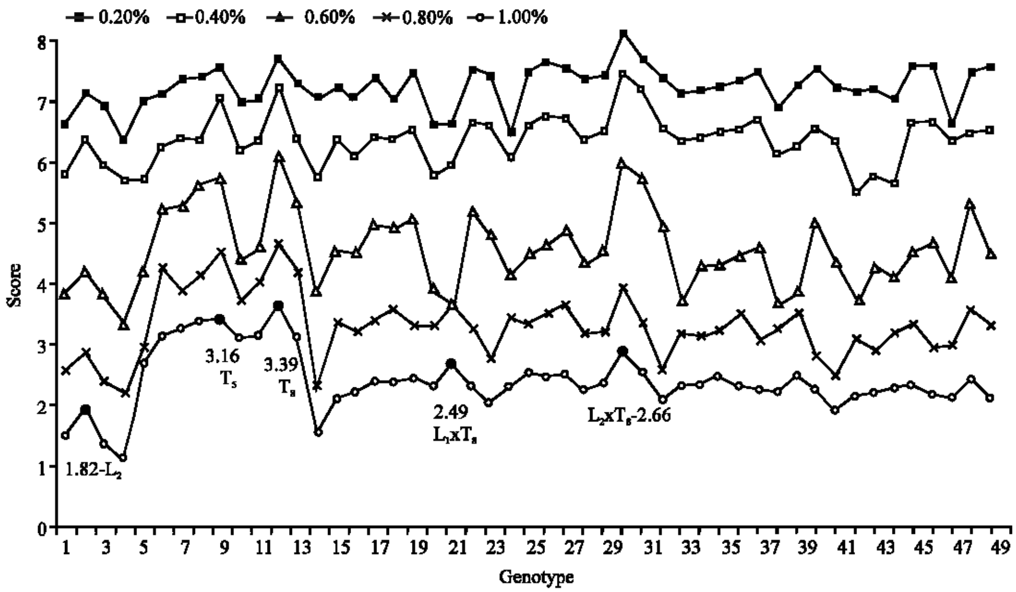

Fig. 2: Effect of NaCl on callus morphology

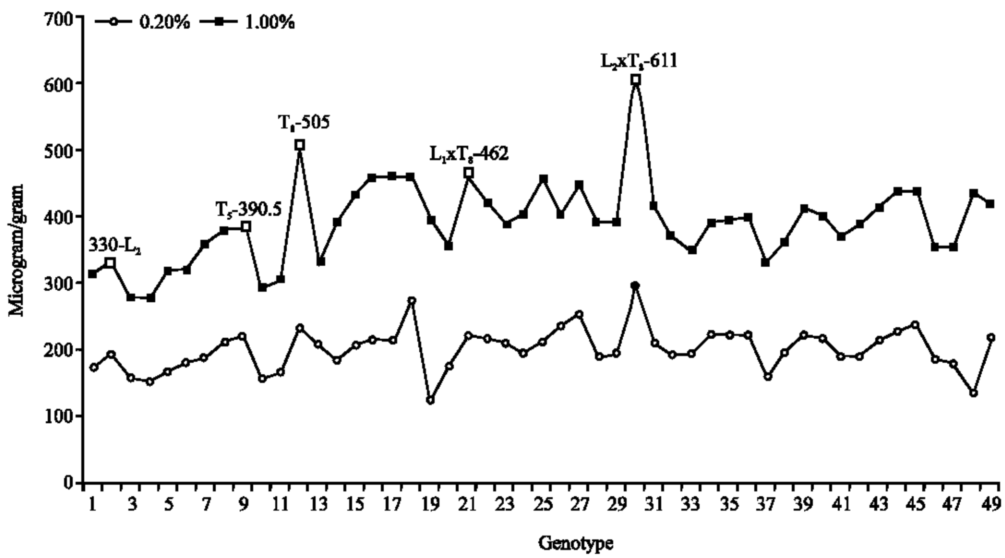

Fig. 3: Effect of NaCl on proline content of callus

various scientists. Laxminarayanan (2000) estimated high levels of proline in media supplemented with increased level of NaCl and suggested as a factor conferring salt tolerance. In contrast, proline accumulation had been excluded as the mechanisms of tolerance in some cases (Dix and Pearce, 1981). Hence the hybrids TS 29/BTS 24, TS 6/BTS 24, IR 58025 A/Vytilla 1 and TS 29/Pokkali could be adjudged as superior ones for salt tolerant conditions as they showed less reduction in callus weight and high callus morphology scores and proline accumulation even at high levels of NaCl concentrations.

Combining *in vitro* and *in vivo* studies, it could be concluded that the hybrids TS 29/BTS 24, TS 6/BTS 24 and IR 58025 A/Vytilla 1 were stable and salt tolerant hybrids along with TS 6/Vytilla 1 and TS 6/TRY 1. Hence these hybrids could be recommended for saline environments or could be used further to develop salt tolerant somaclones.

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